Cell Stress & Chaperones (1996) 1 (4), 251–260

Diminished heat shock response in the aged myocardium

Marius Locke[1] and Robert M. Tanguay[2]

[1]School of Physical and Health Education, University of Toronto, 320 Huron Street, Toronto, Ontario, M5S 3J7 Canada
[2]Centre de recherche du CHUL and Laboratoire de Génétique Cellulaire et développementale, RSVS, Pavillon C.E. Marchand, Université Laval, Ste Foy, Québec, G1K 7P4 Canada

Abstract Induction of heat shock proteins (Hsps), Hsp72 in particular, has been associated with myocardial protection. Since a decreased Hsp response has been reported to occur with aging, it was of interest to determine if hearts from aged animals also demonstrate an altered heat shock response and subsequent myocardial protection. Adult (6 months old) and aged (22 months old) Fischer 344 rats were heat stressed by raising their rectal temperatures to 41°C for 10 min. At selected times following heat stress (0–24 h) hearts were examined for heat shock transcription factor trimerization and DNA-binding activity (Hsf1 activation), Hsp72 mRNA accumulation, Hsp72 and Hsf1 protein content, as well as, protection from ischemia using the Langendorff isolated heart model. Following heat stress, hearts from aged animals demonstrated a 47% reduction in Hsf1 activation, a reduction in Hsp72 mRNA and a 35% reduction in Hsp72 protein content, compared to hearts from adults. Interestingly, myocardial Hsf1 protein content was similar between aged and adult animals. Hearts from heat stressed adult animals (24-h prior) demonstrated an enhanced postischemic recovery as indicated by a greater recovery of left ventricular pressure and rate of contraction ($P < 0.05$), while hearts from heat stressed aged animals failed to demonstrate an enhanced postischemic recovery. These results suggest that hearts from aged animals exhibit an impaired ability to produce the protective Hsps and thus, may explain, at least in part, the increased susceptibility of aged hearts to stress.

INTRODUCTION

A number of biochemical and physiological processes decline with age, rendering aged organisms more susceptible to certain stresses, including heat. Indeed, in humans, a strong correlation between age and mortality from hyperthermia exists (Shock et al 1984). A decreased ability of vital organs, such as the myocardium, to withstand episodes of stress may be crucial to survival of the entire organism. At the cellular level, it is well known that all cells respond to heat and other stresses by the rapid synthesis of 'stress' or 'heat shock proteins' (Hsps). While the exact function of certain Hsps remains unknown, they have been shown to protect cells from stress (Johnston and Kucey 1988; Riabowol et al 1988; Landry et al 1989; Angelides et al 1991; Li et al 1991, 1992).

Received 22 April 1996; Revised 8 July 1996; Accepted 10 July 1996

Correspondence to: Marius Locke, Tel: +1 416 978 7055; Fax: +1 416 978 4384; E-mail: Locke@PHE.utoronto.ca

In the myocardium, an elevated myocardial Hsp expression has been associated with an enhanced postischemic recovery (Currie et al 1988; Currie and Karmazyn, 1990; Karmazyn et al 1990; Locke et al 1995b) and a reduction in infarct size (Donnelly et al 1992; Currie et al 1993; Hutter et al 1994) (for reviews see Knowlton 1995, Mestril and Dillmann 1995). More recently, transgenic mice expressing either the rat (Marber et al 1995) or human (Plumier et al 1995) Hsp72 transgene products in their myocardium, have been shown to demonstrate a reduced infarct size and an enhanced postischemic recovery compared to non-transgenic controls. These studies strongly suggest that expression of Hsps, Hsp72 in particular, provides protection to the heart during episodes of stress.

Stress-induced transcriptional regulation of Hsp72 and other Hsps is mediated by activation and binding of the heat shock transcription factor (Hsf1) to a specific DNA sequence located upstream from all Hsp genes, known as the heat shock element (HSE) (Amin et al 1988). In the unstressed state, Hsf1 exists as inactive

non-DNA-binding monomers, but following exposure to protein damaging stresses, Hsf1 monomers assemble into DNA binding trimers that bind to the HSE (Sarge et al 1993). Cells from aged animals have been shown to demonstrate a diminished heat shock protein induction (Liu et al 1989; Choi et al 1990; Fargnoli et al 1990; Blake et al 1991; Heydari et al 1993; Nitta et al 1994). For example, Heydari et al (1993) has shown that heat shock induction of Hsp72 is reduced 50% in hepatocytes from aged rats. Thus, it was of interest to determine if the myocardium from aged animals also demonstrates an altered heat shock response and subsequent myocardial protection.

MATERIALS AND METHODS

Animals and heat shock

Adult (5–6 months) and aged (21–22 months) barrier reared male Fischer 344 rats (Harlan/Sprague-Dawley Inc., Indianapolis, IN) were used in these experiments. All experiments and procedures were conducted according to NIH guidelines and were approved by the Animal Care Committee of the Deborah Research Institute. Animals were maintained on a 12-h dark/light cycle, housed at 20 +1°C, 50% relative humidity and fed and watered ad libitum. Animals subjected to heat shock were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and placed on a heating pad set at 55°C until rectal temperature reached 0.5°C below the desired temperature. The animal was then placed on a Harvard Homeothermic Blanket and body temperature maintained at the desired temperature (40, 41 or 42°C). During heat shock, rectal temperature was carefully maintained for 10 min within 0.5°C of the desired temperature and was monitored prior to, during and after heat shock by a rectal probe. For experiments that required recovery after heat shock, a 10 min 41°C heat shock was selected, since it was observed that the aged animals could not survive a 15 min, 42°C heat shock. Following the 10-min 41°C heat shock, animals were cooled and revived by the oral administration of water. At selected times following heat shock (1, 3 and 24 h), animals were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and the heart quickly removed and either snap frozen in liquid nitrogen or placed in ice cold saline and cannulated for hemodynamic assessment (Langendorff technique).

Preparation of protein extracts

Protein extracts were prepared according to the method of Mosser et al (1988). Briefly, portions of the left ventricle were thawed and homogenized in 15 volumes of extraction buffer (25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA (pH 8.0) 20 mM HEPES (pH 7.9) 0.5 mM DTT, 0.5 mM phenylmethylsufonylfluoride) at 4°C at 5000 rpm using a Polytron. Tissue lysates were centrifuged at 14 000 rpm at 4°C (16 000 × *g*) for 20 min in an Eppendorf centrifuge. The supernatant was removed and stored at –70°C. Protein concentrations were determined by the Bradford method (1976).

Mobility shift analyses

Protein extracts (50 μg) from control and heat shocked rat hearts were incubated with an ^{32}P-labelled self-complementary ideal HSE oligonucleotide (5′-CTA GAA GCT TCT AGA AGC TTC TAG-3′) in binding buffer (10% glycerol, 50 mM NaCl, 1.0 mM EDTA (pH 8.0) 20 mM Tris (pH 8.0) 1.0 mM DTT, 0.3 mg/ml BSA) with 0.1 ng (50 000 cpm) of ^{32}P labelled oligonucleotide and 5.0 μg poly (dI dC) (Pharmacia Fine Chemicals, Piscataway, NJ) for 30 min at room temperature. Samples were electrophoresed on 4% acrylamide gel at 200 volts for 2–3 h. Gels were dried and exposed to X-ray film (Amersham-ECL). Hsf1 activation was assessed by band shifts and correct Hsf1–HSE interaction was confirmed by incubating extracts with a 200-fold molar excess of unlabelled HSE as previously described (Locke et al 1995a).

RNA isolation and Northern blot analyses

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi 1987). Ten micrograms of total RNA was separated on a 1% formaldehyde agarose gel, transferred to nitrocellulose (0.22 μm thickness, Bio-Rad Laboratories) and fixed by baking at 80°C for 4 h. Blots were prehybridized at 48°C in 50% de-ionized formamide, 0.1% sodium dodecyl sulfate (SDS), 5X SSPE, 5X Denhardt's solution and 50 μg/ml of denatured salmon sperm DNA for 4–6 h. Blots were probed with a 1.7 kb EcoR1 fragment of the human *hsp70* gene (Adams et al 1992) labelled with ^{32}P-CTP using the random prime method (Feinberg and Volgelstein 1983). Hybridization was performed at 48°C in 50% de-ionized formamide, 0.1% SDS, 5X SSPE, 2X Denhardt's solution and 50 μg/ml of denatured salmon sperm DNA. Blots were washed with 0.1 × SSC, 0.1% SDS at 48°C and exposed to Kodak X-OMAT LS film at –70°C. After autoradiography, blots were stripped and reprobed with an ^{32}P-ATP end labelled 26 bp oligonucleotide specific for a fragment of the 28S rRNA as described by Barbu and Dautry (1989).

Polyacrylamide gel electrophoresis and immunoblotting

Muscle portions were homogenized in 600 mM NaCl, 15 mM Tris pH 7.5 and protein concentration determined by the method of Lowry et al (1951). One

dimensional (1-D) SDS polyacrylamide gel electrophoresis (PAGE) was performed according to the method described by Laemmli (1970), except that the separating gel (0.15 × 4.5 × 8 cm) consisted of a 5–15% polyacrylamide gradient. Following electrophoretic separation, proteins were transferred to nitrocellulose membranes (0.22 μm thickness, Bio-Rad Laboratories) as described by Towbin et al (1979) using the Bio-Rad mini-protean II gel transfer system. Following protein transfer, blots were reacted with a polyclonal antibody *799* (diluted 1: 2500) specific for the inducible member of the Hsp70 family, (Hsp72; Currie et al 1993; Tanguay et al 1993) as previously described (Locke et al 1995a). For identification of the Hsf1 protein, a commercially available antibody specific for the Hsf1 protein (PA3–017, Affinity Bioreagents Inc., Golden, CO) was used. Blots were placed in Hsf1 antibody diluted 1: 500 in TTBS supplemented with 2% (w/v) non-fat dried milk powder for 3 h. Blots were washed three times (5 min each) in TTBS, and placed in a solution of a 1:10 000 dilution of secondary antibody consisting of goat-anti-rabbit IgG conjugated to alkaline phosphatase for 1 h. Blots were washed three times (5 min each) in TTBS, twice (5 min each) in assay buffer (100 mM diethanolamine, 1 mM $MgCl_2$, pH 10.0). A Western Exposure™ Chemiluminescent Detection System (Clontech Laboratories Inc., Palo Alto, CA) was used to visualize the Hsf1 protein according to the manufacturer's instructions. Blots were then wrapped in plastic wrap and exposed to Kodak X-OMAT LS film. To determine non-specific binding, duplicate gels were run, transferred and reacted, but the primary antibody was omitted. Quantitation of bands from immunoblots or exposed film was performed by scanning blots with a Shimadzu CS-9000U densitometer.

Isolated heart preparation

Twenty-four hours after heat shock, rats were anesthetized with pentobarbital (60 mg/kg, i.p.), injected with 1000 units of heparin (via tail vein), and their hearts rapidly removed for evaluation of cardiac function in vitro. The aorta was cannulated and the coronaries were perfused by the Langendorff technique under constant pressure conditions (50 mmHg); the hearts were electrically paced at 320 beats/min. A fluid-filled balloon-tipped catheter inserted into the left ventricle was used to assess left ventricular function (left ventricular developed pressure, LVDP; left ventricular +dP/dt). The balloon volume was inflated to 50 μl. Previous experiments have shown that this volume corresponds to the plateau region of the Starling curve (Klabunde and Coston 1995). Coronary flow (CF) was recorded using a Transonic flowmeter probe placed on the perfusion tubing. Hearts were perfused with a Krebs–Henseleit solution (4.7 mM KCL, 2.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM Mg_2SO_4, 118 mM NaCl, 25 mM $NaHCO_3$ and 11 mM glucose) bubbled with a gas mixture of 95% O_2/5% CO_2 to maintain a pH of 7.4 at 37°C.

The protocol for these isolated heart experiments was as follows: following a 45-min equilibration period, hearts were subjected to 30 min of complete, global ischemia by stopping the coronary perfusion and electrical pacing. At the end of this ischemic period, the CF and electrical pacing was re-established for 30 min. Hearts that fibrillated excessively (> 2 min) during reperfusion were excluded from the study. Data were recorded continuously throughout the protocol using a MacLab. Data were evaluated at 5 min prior to the ischemic period and at 0, 5, 10, 15, 20, 25 and 30 min during reperfusion.

Statistical analyses

Data obtained from four groups (immunoblots) were analyzed by analysis of variance followed by Tukey's post hoc test, while comparisons between two groups (Langendorff technique) were analyzed using an unpaired Student's *t*-test. In all cases, the level of significance was set at $P < 0.05$.

RESULTS

Physical characteristics and thermal response during heat shock

Aged animals demonstrated a greater heart weight ($P < 0.05$) and body weight ($P < 0.05$) than adult animals (Table 1). However, comparison of heart weight to body weight ratios demonstrated no differences between adult and aged animals.

No differences were detected in the thermal responses between adult and aged animals. Preheat shock rectal temperatures were 37.1 ± 0.13°C for adult animals and 36.9 ± 0.13°C for aged animals, while peak temperatures were 41.4 ± 0.06°C for adult animals and 41.3 ± 0.1°C for aged animals. Heating rates for adult and aged animals were similar (0.31 ± 0.01°C/min vs 0.31 ± 0.01°C/min, respectively) as were cooling rates (0.19 ± 0.02°C/min for adults vs 0.20 ± 0.02°C/min for aged). These results indicate that adult and aged animals experienced a similar thermal stress during heat shock.

Hsp72 protein accumulation is diminished in the aged heart following heat shock

To determine Hsp72 content in the hearts from unstressed and heat shocked (24 h after a 10-min 41°C heat shock) adult and aged animals ($n = 5$ for both

Table 1 Physical characteristics and thermal responses of adult and aged Fischer 344 rats

	Adult	Aged
Body weight (g)	358.3 ± 4.37	445.9 ± 5.28*
Heart weight (mg)	961 ± 19	1150 ± 28*
Heart weight/Body weight ratio (mg/g)	2.57 ± 0.06	2.53 ± 0.05
Preheat shock temperature (°C)	37.1 ± 0.13	36.9 ± 0.12
Peak temperature (°C)	41.4 ± 0.06	41.3 ± 0.07
Heating rate (°C/min)	0.31 ± 0.01	0.31 ± 0.01
Cooling rate (°C/min)	0.19 ± 0.02	0.20 ± 0.02

Data are expressed as mean ± SEM. Statistically significant differences ($P < 0.05$) from the adult values are indicated (for all groups the minimum number of animals was 8).

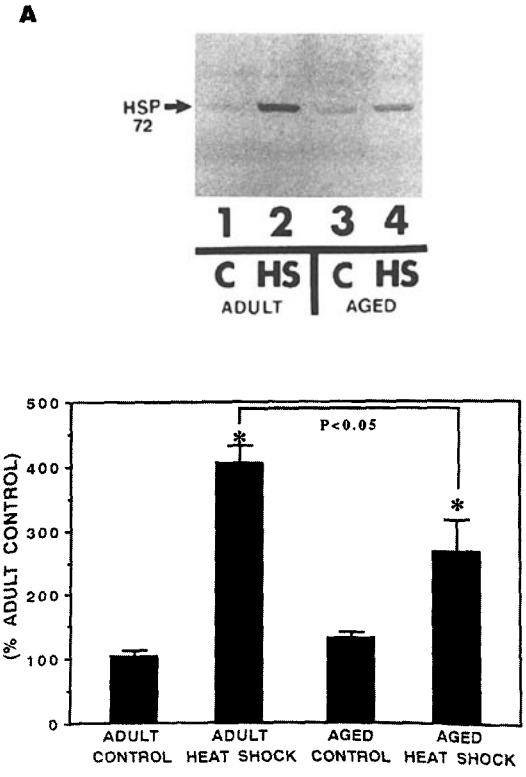

Fig. 1 Hsp72 content is increased in the adult and aged rat heart 24 h following a 10 min 41°C heat shock. (A) Proteins from the left ventricle were separated by SDS-PAGE, transferred to nitrocellulose and reacted with an Hsp72-specific antibody. Lane 1: adult control, lane 2: adult heat shocked, lane 3: aged control, lane 4: aged heat shocked. (B) Graphical representation of the densitometric scans from similar blots presented in (A). Data are expressed as mean + SEM. Statistical significance between adult heat shocked and aged heat shocked groups is indicated ($n = 5$ for all groups).

groups), portions of the left ventricle were analyzed for Hsp72 (the inducible member of the Hsp70 family) content by Western blotting. A representative Western blot containing the SDS-PAGE separated heart proteins reacted with antibody for Hsp72 is shown in Figure 1A. Hsp72 content of hearts from adult and aged control animals was low (Fig. 1, lanes 1 and 3) and when similar blots were quantified by densitometric scanning, no statistically significant difference in Hsp72 content was detected between hearts from adult controls and aged controls (Fig. 1B). Hsp72 content in hearts from adult animals that were heat shocked 24 h prior, was elevated 4-fold compared to adult controls ($P < 0.05$)(Fig. 1A, lane 2 and Fig. 1B), while only a 2.6-fold increase in Hsp72 content was observed in hearts from heat shocked aged animals ($P < 0.05$)(Fig. 1A, lane 4 and Fig. 1B). When the myocardial Hsp72 content of heat shocked adult and aged animals was compared, the amount of Hsp72 in the aged heart was reduced 35% ($P < 0.05$) compared to the adult heat shocked heart (Fig. 1A, lanes 2, 4 and Fig. 1B). These results suggest that the aged heart demonstrates a decreased accumulation of Hsp72 when challenged with heat stress.

Hsp72 mRNA accumulation is diminished in the aged heart following heat shock

To determine myocardial Hsp72 mRNA accumulation following heat shock, Hsp70 mRNA was assessed by Northern blotting. Hsp70 mRNA was not detected in hearts from unstressed adult or aged animals (Fig. 2A, lanes 1 and 5). Directly, following the 10-min heat shock at either 40, 41 or 42°C, at least three mRNA species with molecular masses of 2.7, 3.1 and 3.3 kb were detected in hearts from both adult and aged animals (Fig. 2A, lanes 2–4 and 6–8). However, hearts from aged animals demonstrated a reduced accumulation of all Hsp70 mRNAs at both 40 and 41°C (Fig. 2A, lanes 6–8) compared to hearts from adult animals. Following a 42°C heat shock Hsp70 mRNA demonstrated a similar accumulation in the hearts from adult and aged animals (Fig. 2A, lanes 4 and 8). However, aged animals were unable to survive this severe stress. The 3.1 and 3.3 kb species have been shown to correspond to Hsp72, the inducible Hsp70 isoform, while the 2.7 kb species corresponds to Hsc73, the constitutive Hsp70 isoform (Locke et al 1995a).

To determine if the induction of Hsp70 mRNAs follows different kinetics in hearts from aged animals, Hsp70 mRNA content was also assessed at 1 and 3 h after a 10 min 41°C heat shock. The amount of myocardial Hsp70 mRNA was slightly reduced at 1-h post heat shock (Fig. 2B, lane 7) in aged animal and by 3-h post heat shock, Hsp72 mRNA was undetectable in hearts from both adult

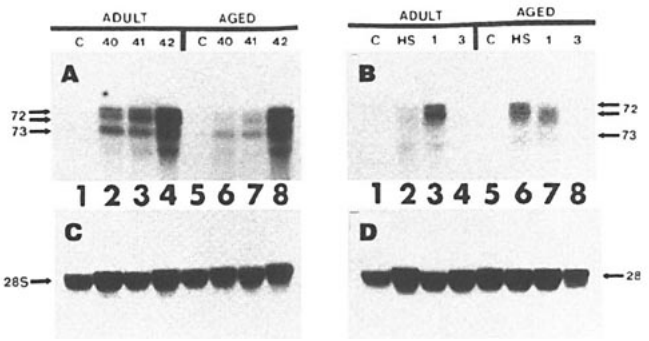

Fig. 2 Hsp72 mRNA content is decreased in the aged rat heart following heat shock. Hearts were removed from animals directly after heat shock or at 1- and 3-h post heat shock. RNA was isolated, separated, transferred to a nylon membrane and hybridized with a ^{32}P-labelled cDNA probe for Hsp72 as described in Materials and Methods. Shown here is the autoradiogram. (A) Lane 1: control (unstressed) adult rat heart, lane 2: adult rat heart after a 10 min/40°C heat shock, lane 3: adult rat heart after a 10 min/41°C heat shock, lane 4: adult rat heart after a 10 min/42°C heat shock, lane 5: control (unstressed) aged rat heart, lane 6: aged rat heart after a 10 min/40°C heat shock, lane 7: aged rat heart after a 10 min/41°C heat shock, lane 8: aged rat heart after a 10 min/42°C heat shock. (B) Lane 1: control (unstressed) adult rat heart, lane 2: adult rat heart after a 10 min/41°C heat shock, lane 3: adult rat heart 60 min after a 10 min/41°C heat shock, lane 4: adult rat heart 180 min after a 10 min/41°C heat shock, lane 5: control (unstressed) aged rat heart, lane 6: aged rat heart after a 10 min/41°C heat shock, lane 7: aged rat heart 60 min after a 10 min/41°C heat shock, lane 8: aged rat heart 180 min after a 10 min/41°C heat shock. (C and D) The blots in panels (A) and (B) reprobed with a ^{32}P end-labelled oligonucleotide, specific for a fragment of the 28S rRNA.

and aged animals (Fig. 2B, lanes 4 and 8). To demonstrate loading of total RNA, blots were stripped and reprobed with an end-labelled oligonucleotide specific for the 28S ribosomal fragment (Fig. 2C, D). These results suggest that although the kinetics of Hsp70 mRNA induction in the adult and aged rat heart is similar following heat shock, Hsp70 mRNA accumulation in the aged heart is reduced.

Hsf1 activation is reduced in the aged heart

The term Hsf1 activation is used to describe the Hsf1 trimer binding to the HSE. Hsf1 activation in protein extracts from control and heat-shocked adult and aged rat hearts was assessed by mobility shift (MS-PAGE). No Hsf1 activation was detected in extracts from control adult or aged rat hearts (Fig. 3, lanes 2 and 6, respectively). Hsf1 activation was detected in extracts from adult rat hearts that were heat shocked for 10 min to either 40, 41 or 42°C (Fig. 3, lanes 3, 4 and 5, respectively) and in extracts from aged rat hearts that were heat shocked to either 40, 41 or 42 °C for 10 min (Fig. 3, lanes 7, 8 and 9, respectively). However, the level of Hsf1 activation in the aged rat hearts at any given temperature was reduced considerably when compared to the adult heart. When Hsf1 bands from similar autoradiograms were quantified by densitometry, the level of Hsf1 activation was significantly reduced ($P < 0.05$) in the aged heart and was only 53% of the adult heart (Fig. 4).

To determine if the time course of Hsf1 activation and inactivation differed in the aged heart, Hsf1 activation was also assessed at 1- and 3-h post heat shock. MS-PAGE analyses of heart extracts from adult and aged rats that were heated to 41°C and allowed to recover, demonstrates that Hsf1 activation was only slightly detectable at 1-h post heat shock in both adult and aged rat hearts (Fig. 5, lanes 4 and 8, respectively). By 3-h post heat shock, Hsf1 activation was undetectable in both adult and aged rat hearts (Fig. 5, lanes 5 and 9, respectively). These results demonstrate that the kinetics of Hsf1 inactivation are similar in both the adult and aged rat heart.

Hsf1 protein content is similar in the aged and adult heart

To determine if the reduced heat shock response in the aged hearts is the result of a decreased amount of Hsf1

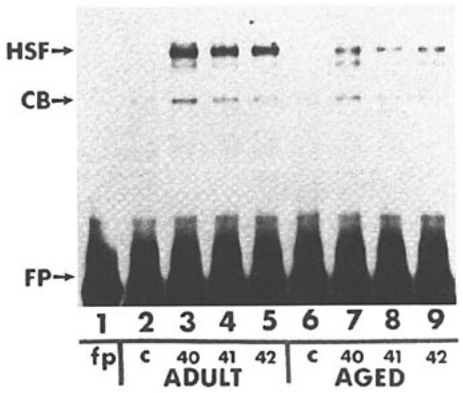

Fig. 3 Heat shock factor activation is reduced in aged rat hearts following heat shock. Hearts were removed from animals directly after heat shock. Protein extracts were incubated with a ^{32}P-labelled heat shock element and analyzed by MS-PAGE as described in Materials and Methods. Lane 1: free probe, lane 2: unstressed (control) adult rat heart, lane 3: adult rat heart after a 10 min/40°C heat shock, lane 4: adult rat heart after a 10 min/41°C heat shock, lane 5: adult rat heart after a 10 min/42°C heat shock, lane 6: unstressed (control) aged rat heart, lane 7: aged rat heart after a 10 min/40°C heat shock, lane 8: aged rat heart after a 10 min/41°C heat shock, lane 9: aged rat heart after a 10 min/42°C heat shock. Hsf = heat shock transcription factor complex, CB = constitutive heat shock binding, FP = free probe.

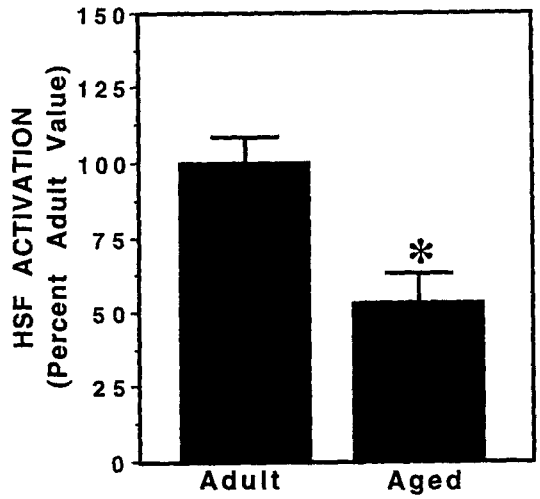

Fig. 4 Graphical representation of the densitometric scans from similar autoradiograms to that presented in Figure 3. Data are expressed as mean ± SEM. Statistical significance is indicated ($n = 5$ for both groups).

protein, Hsf1 content was determined by Western blotting. A representative Western blot containing the SDS-PAGE separated myocardial proteins from adult and aged animals, reacted with Hsf1 antibody is shown in Figure 6A. The amount of Hsf1 detected in aged and adult hearts was visually similar. Two non-specific bands were identified by running identical blots but with omission of the Hsf1 antibody (Fig. 6A). When a

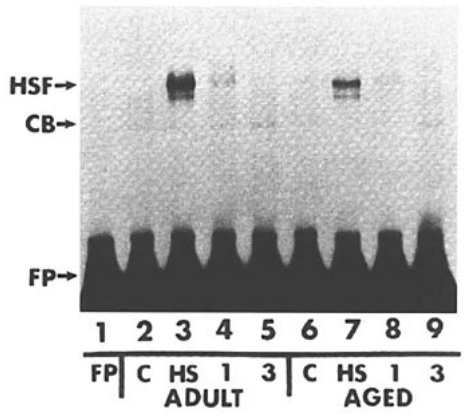

Fig. 5 Hsf1 inactivation follows similar kinetics in the aged and adult heart. Protein extracts were incubated with a ^{32}P-labelled heat shock element and analyzed by MS-PAGE as described in Materials and Methods. Lane 1: free probe, lane 2: unstressed (control) adult rat heart, lane 3: adult rat heart after a 10 min/41°C heat shock, lane 4: adult rat heart 60 min after a 10 min/41°C heat shock, lane 5: adult rat heart 180 min after a 10 min/41°C heat shock, lane 6: unstressed (control) aged rat heart, lane 7: aged rat heart after a 10 min/41°C heat shock, lane 8: aged rat heart 60 min after a 10 min/41°C heat shock, lane 9: aged rat heart 180 min after a 10 min/41°C heat shock. Hsf = heat shock transcription factor complex, CB = constitutive heat shock binding, FP = free probe.

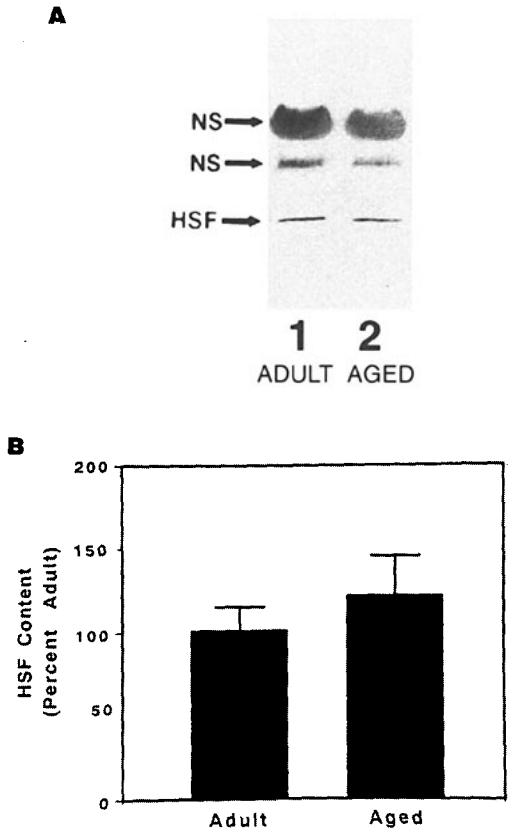

Fig. 6 Hsf1 content is similar in the adult and aged rat heart. (A) Proteins from the left ventricle were separated by SDS-PAGE, transferred to nitrocellulose and reacted with an Hsf1-specific antibody as described in Materials and Methods. Lane 1: unstressed (control) adult rat heart, lane 2: unstressed (control) aged rat heart. (B) Graphical representation of the densitometric scans from similar autoradiograms presented in (A). Data are expressed as mean ± SEM. No statistically significant differences were detected. The position of the heat shock transcription factor (Hsf) is indicated by an arrow. The two upper bands are non specific (NS) as shown by incubation in the absence of primary antibody.

Table 2 Absolute values for coronary flow (CF), rate of contraction (+dP/dt) and left ventricular developed pressure (LVDP)

	CF (ml/min)	+dP/dt (mmHg/sec)	LVDP (mmHg)
Adult Control	6.86 ±0.67	842 ±116	71.91 ±2.57
Heat shocked	6.25 ±0.29	650 ±34.9	63.36 ±3.02
Aged Control	7.51 ±0.71	567 ±24.6	55.69 ±2.94
Heat shocked	8.04 ±0.80	566 ±63.4	55.65 ±5.99

No statistically significant differences were detected between hearts from heat shocked animals and age matched controls. ($n = 7$ for all groups except adult heat shocked, where $n = 6$).

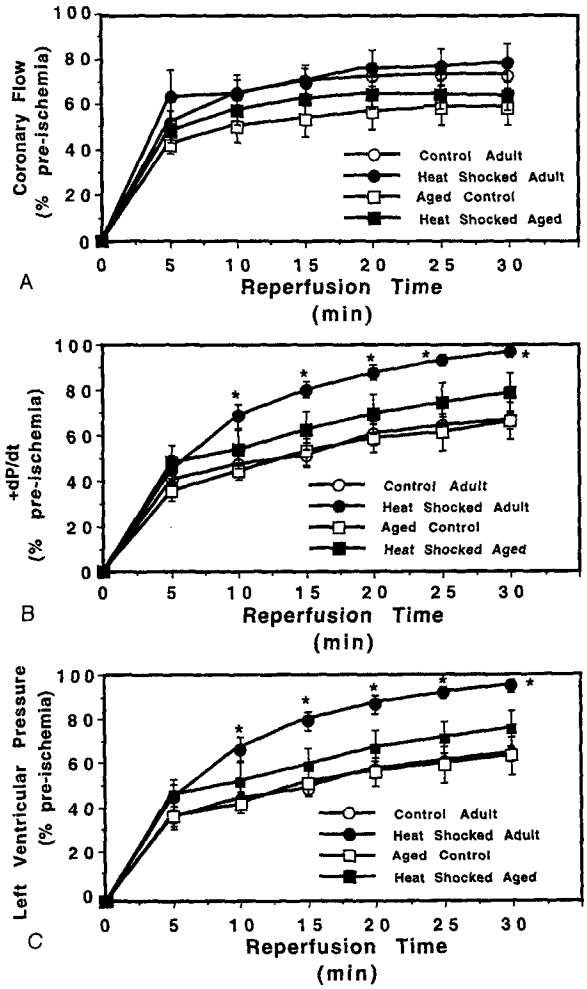

Fig. 7 Heat shock does not enhance the postischemic recovery of the rate of contraction or the development of left ventricular pressure in the aged rat heart. (A) Postischemic coronary flow during reperfusion. (B) Rate of contraction during reperfusion. (C) Left ventricular developed pressure during reperfusion. Data are expressed as a percentage of the preischemic value (mean ± SEM). Statistically significant differences from age-matched controls are indicated. For all panels: solid circles = heat shocked adults, open circles = control adults, solid squares = heat shocked aged, open squares = control aged.

similar blot to that shown in Figure 6A was quantified by densitometric scanning, the Hsf1 content of hearts from aged animals was similar (Fig. 6B) to the amount in hearts from adult animals. These results demonstrate that the amount of Hsf1 protein in the aged and adult heart is similar.

Heat shock protects the adult heart but not the aged heart

To determine if the reduced heat shock response in the aged heart is associated with a reduced protection from ischemia, hearts from control and heat shocked adult and aged animals were evaluated for hemodynamic function after 30 min of global ischemia using the Langendorff isolated heart model. Table 2 shows the pre-ischemic absolute values for CF, rate of contraction (+dP/dt) and LVDP for control and heat shocked (10 min 41°C 24-h prior) adult and aged hearts. No significant differences in absolute values for CF, +dP/dt or LVDP were detected for either adult or aged heat shock treated animals when compared to age-matched controls.

No statistically significant differences in normalized CF values (% preischemia) were detected between adult or aged animals when compared to age-matched controls at any time during reperfusion (Fig. 7A). However, hearts from heat shocked adult animals demonstrated a more rapid recovery of rate of contraction (+dP/dt) of the left ventricle (Fig. 7B) compared to hearts from adult controls. Hearts from heat shocked adult animals demonstrated a statistically significant difference ($P < 0.05$) in left ventricular +dP/dt at 10 min of reperfusion (68.5 ± 5.0% vs 47.8 ± 4.5% for controls) compared to adult controls. This pattern of left ventricular +dP/dt recovery continued during the reperfusion period, such that, at 30 min of reperfusion, hearts from heat shocked adult animals recovered 97.1 ± 2.7% of preischemic left ventricular +dP/dt ($P < 0.05$). In contrast, hearts from heat shocked aged animals demonstrated no significant difference in recovered left ventricular +dP/dt compared to aged controls, at any time during reperfusion.

During reperfusion, hearts from adult heat shocked animals also recovered LVDP to a greater extent than hearts from adult controls. When expressed as a percentage of the pre-ischemic value (Fig. 7C), hearts from heat shocked adult animals demonstrated a significant difference ($P < 0.05$) in LVDP at 10 min of reperfusion (68.9 ± 5.7% vs 46.2 ± 3.5% for adult controls). Compared to adult controls, hearts from heat shocked adult animals demonstrated statistically significant differences in LVDP throughout the reperfusion period, such that, at 30 min of reperfusion, hearts from heat shocked adult animals recovered 97.2 ± 2.7% of preischemic LVDP ($P < 0.05$), while controls recovered only 66.2 ± 3.4% of preischemic LVDP. When compared to aged controls, hearts from heat shocked aged animals demonstrated no significant difference in recovered LVDP at any time during reperfusion, although LVDP values tended to be above control values throughout the reperfusion period. These results suggest that heat

shock provides an enhanced postischemic recovery to the adult myocardium but does not provide an enhanced postischemic recovery to the aged myocardium.

DISCUSSION

The novel features of the present data are that following heat shock, hearts from aged animals demonstrate a reduced Hsf1 activation and reduced Hsp72 expression compared to hearts from adult animals. In addition, 24 h after heat shock, hearts from aged animals failed to demonstrate an enhanced post-ischemic recovery. In the adult rat heart, heat shock and the concomitant induction of the 'protective' Hsps, Hsp72 in particular, has been associated with an enhanced postischemic recovery (Currie et al 1988; Currie and Karmazyn 1990; Karmazyn et al 1990; Locke et al 1995b) and a reduction in infarct size (Donnelly et al 1992; Currie et al 1993; Hutter et al 1994). The present study supports this concept, since heat shock and the induction of Hsp72, was associated with protection to hearts from adult animals. In contrast, hearts from heat shocked aged animals, failed to demonstrate any significant increase in myocardial protection. Whether the lack of myocardial protection observed in the heat shocked aged heart is directly related to the reduced Hsp72 expression or some other aspect(s) of aging or heat shock remains to be determined.

Although the Hsp72 response following heat shock, was reduced in aged animals compared to adult animals, it was significantly elevated (2.6-fold) above adult control values. Since previous work has shown a direct correlation between Hsp72 content and protection (Hutter et al 1994; Locke et al 1995b), some protection should be expected in the heat shocked aged animal. Indeed, during reperfusion, although hearts from heat shocked aged animals failed to demonstrate a statistically significant difference, a trend towards an elevation in both +dP/dt and LVDP was observed compared to aged controls. Thus, it may be the case that some 'critical amount' of Hsp72 may be required to provide protection. The present study and others (Donnelly et al 1992; Currie et al 1993; Hutter et al 1994; Locke et al 1995b) have assessed Hsp72 content by Western blotting. This technique measures only total Hsp72 content and does not distinguish between intracellular 'free' and 'bound' forms of the protein. It remains conceivable then, that following heat stress, only the 'free' form of Hsp72 would be available to restore and/or repair proteins that are damaged during ischemia. Thus, although Hsp72 content was elevated in the aged heart following heat shock, the amount of Hsp72 content in the 'bound' and 'free' forms remains unknown.

It is well established that aged cells demonstrate a decreased Hsp induction (Liu et al 1989; Choi et al 1990; Fargnoli et al 1990; Blake et al 1991; Heydari et al 1993; Nitta et al 1994; Kregal et al 1995). For example, Heydari et al (1993) reported a reduced Hsf1 activation in hepatocytes from aged rats following heat shock. In addition, the induction of Hsp72 was reduced 50%, compared to heat shocked hepatocytes from adult animals. A decreased Hsp response has also been reported in the aged myocardium (Nitta et al 1994; Kregal et al 1995) as a diminished accumulation of Hsp72 mRNA and protein, have been shown to occur after ischemic (Nitta et al 1994) and heat stresses (Kregal et al 1995), respectively. However, in neither study, were the mechanisms for the reduced Hsp response examined.

The results from our study agree with previous studies (Liu et al 1989; Choi et al 1990; Fargnoli et al 1990; Blake et al 1991; Heydari et al 1993; Nitta et al 1994; Kregal et al 1995) since both a reduced Hsp72 mRNA accumulation and reduced Hsp72 protein were detected in the myocardium of aged animals following heat shock. In addition, in this study, Hsf1 activation was also shown to be reduced 47% in the heat shocked aged heart, yet there was no difference in Hsf1 protein content between adult and aged rats hearts. This suggests that the aged myocardium contains a sufficient amount of the Hsf1 protein, but exhibits an impaired ability to form the necessary DNA binding competent trimers that are required for stress induction of the *hsp72* gene. A similar relationship between Hsf1 activation and Hsf1 content has been reported in the adrenal glands of aged rats following restraint induced stress (Fawcett et al 1994). The exact reason(s) and mechanism(s) for the impaired Hsf1 activation in aged cells remains to be determined. However, taken together, these data suggest that an impairment in translating stress signals into the biochemical steps necessary for induction of the stress response accompanies aging.

In conclusion, the present study suggests that following heat shock, the Hsf1 activation process in the aged heart is impaired, resulting in a decreased Hsp expression and subsequent loss of myocardial protection. The decreased ability to mount a stress response, and synthesize a specific quantity of Hsps, may explain, at least in part, the increased susceptibility of aged hearts to physiologically relevant stresses, such as ischemia, hyperthermia or strenuous exercise.

ACKNOWLEDGMENTS

The work was supported by Deborah Research Institute Grant 94–15P, a Medical Research Council of Canada Grant to RMT (MT-11086) and a Natural Science and Engineering Research Council of Canada Grant (OGP0183742) to ML.

REFERENCES

Adams MD, Dubnick M, Kerlavage AR et al (1992) Sequence identification of 2375 human brain genes. Nature **355**, 632–634.

Amin J, Anathan J and Voellmy R (1988) Key features of heat shock regulatory elements. Mol. Cell. Biol. **8**, 3761–3769.

Angelidis CE, Lazaridis I and Pagoulatos GN (1991) Constitutive expression of heat-shock protein-70 in mammalian cells confers thermoresistance. Eur. J. Biochem. **199**, 35–39.

Barbu V and Dautry F (1989) Northern blot normalization with a 28S rRNA oligonucleotide probe. Nucleic Acids Res. **17**, 7115–7116.

Blake MJ, Fargnoli J, Gershon D and Holbrook NJ (1991) Concomitant decline in heat-induced hyperthermia and Hsp70 messenger RNA expression in aged rats. Am. J Physiol. **260** (Reg. Integ. Comp. Physiol 29), R663–R667.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Choi H-S, Lin Z, Li B and Liu AY-C (1990) Age-dependent decrease in the heat-inducible DNA sequence-specific binding activity in human diploid fibroblasts. J. Biol. Chem. **265**, 18005–18011.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156–159.

Currie RW and Karmazyn M (1990) Improved post-ischemic ventricular recovery in the absence of changes in energy metabolism in working rat hearts following heat shock. J. Mol. Cell. Cardiol. **22**, 631–636.

Currie RW, Karmazyn M, Kloc M and Mailer K (1988) Heat-shock response is associated with enhanced postischemic ventricular recovery. Circ. Res. **63**, 543–549.

Currie RW, Tanguay RM and Kingma Jr JG (1993) Heat-shock response and limitation of tissue necrosis during occlusion/reperfusion in rabbit hearts. Circulation **87**, 963–971.

Donnelly TJ, Sievers RE, Vissern FLJ, Welch WJ and Wolfe CL (1992) Heat shock protein induction in rat hearts. A role for improved myocardial salvage after ischemia and reperfusion? Circulation **85**, 769–778.

Fargnoli J, Kunisada T, Fornace Jr AJ, Schneider EL and Holbrook NJ (1990) Decreased expression of heat shock protein 70 mRNA and protein after heat treatment in cells of aged rats. Proc. Natl Acad. Sci. USA **87**, 846–850.

Fawcett TW, Sylvester SL, Sarge KD, Morimoto RI and Holbrook NJ (1994) Effects of neurohormonal stress and aging on the activation of mammalian heat shock factor 1. J. Biol. Chem. **269**, 32272–32278.

Feinberg AP and Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**, 6–13.

Heydari AR, Wu B, Takahashi R, Strong R and Richardson A (1993) Expression of heat shock protein 70 is altered by age and diet at the level of transcription. Mol. Cell. Biol. **13**, 2909–2918.

Hutter MM, Sievers RE, Barbosa V and Wolfe CL (1994) Heat-shock protein induction in rat hearts. A direct correlation between the amount of heat-shock protein and the degree of myocardial protection. Circulation **89**, 355–360.

Johnston RN and Kucey BL (1988) Competitive inhibition of hsp70 gene expression causes thermosensitivity. Science **242**, 1551–1554.

Karmazyn M, Mailer K and Currie RW (1990) Acquisition and decay of heat-shock-enhanced postischemic ventricular recovery. Am. J. Physiol. **259** (Heart Circ. Physiol. 28), H424–H431.

Klabunde RE and Coston AC (1995) Nitric oxide synthase inhibition does not prevent cardiac depression in endotoxic shock. Shock **3**, 73–78.

Knowlton AA (1995) The role of heat shock proteins in the heart. J. Mol. Cell. Cardiol. **27**, 121–131.

Kregal KC, Moseley PL, Skidmore R, Gutierrez JA and Guerriero Jr V (1995) HSP70 accumulation in tissues of heat-stressed rats is blunted with advancing age. J. Appl. Physiol. **97**, 1673–1678.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685.

Landry J, Chrétien P, Lambert H, Hickey E and Weber L (1989) Heat shock resistance conferred by expression of the human *hsp27* gene in rodent cells. J. Cell Biol. **109**, 7–15.

Li GC, Li LG, Liu YK, Mak JY, Chen LL, and Lee WM (1991) Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. Proc. Natl Acad. Sci. USA **88**, 1681–1685.

Li GC, Li L, Liu RY, Rehman M and Lee WM (1992) Heat shock protein hsp70 protects cells from thermal stress even after deletion of its ATP-binding domain. Proc. Natl Acad. Sci. USA **89**, 2036–2040.

Liu A, Lin Y-CZ, Choi H-S, Sorhage F and Li B (1989) Attenuated induction of heat shock gene expression in aging diploid fibroblasts. J. Biol. Chem. **264**, 12037–12045.

Locke M, Noble EG, Tanguay RM, Feild MR, Ianuzzo SE and Ianuzzo CD (1995a) Activation of the heat shock transcription factor in the rat heart following heat shock and exercise. Am. J. Physiol. **268** (Cell Physiol. 37), C1387– C1394.

Locke M, Tanguay RM, Klabunde RE and Ianuzzo CD (1995b) Enhanced post-ischemic recovery following exercise induction of HSP 70 in rat heart. Am. J. Physiol. **269** (Heart Circ. Physiol. 38), H320–H325.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurements with the folin phenol reagent. J. Biol. Chem. **193**, 265–275.

Marber MS, Mestril R, Chi S-H, Sayen MR, Yellon DM and Dillmann WH (1995) Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. J. Clin. Invest. **95**, 1446–1456.

Mestril R and Dillmann WH (1995) Heat shock proteins and protection against ischemia. J. Mol. Cell. Cardiol. **27**, 45–52.

Mosser DD, Theodorakis G and Morimoto RI (1988) Coordinate changes in heat shock element-binding activity and Hsp70 transcription rates in human cells. Mol. Cell. Biol. **8**, 4736–4744.

Nitta Y, Abe K, Aoki M, Ohno I and Isoyama S (1994) Diminished heat shock protein 70 mRNA induction in aged rat hearts after ischemia. Am. J. Physiol. **267** (Heart Circ. Physiol. 36), H1795–H1803.

Plumier J-C, Ross BM, Currie RW, Angelidis CE, Kazlaris H, Kollias G and Pagoulatos GN (1995) Transgenic mice expressing the human heat shock protein 70 have improved postischemic myocardial recovery. J. Clin. Invest. **95**, 1854–1860.

Riabowol KT, Mizzen LA and Welch WJ (1988) Heat shock is lethal to fibroblasts microinjected with antibodies against Hsp70. Science **242**, 433–436.

Sarge KD, Murphy SP and Morimoto RI (1993) Activation of heat shock gene transcription by heat shock factor-1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol. Cell. Biol. **13**, 1392–1407.

Shock NW, Greulich RC, Andres R, Arenberg D, Costa PT, Lakatta EG and Tobin JD (1984) Normal human aging: the Baltimore longitudinal study of aging. U.S. Government Printing Office, Washington, DC.

Tanguay RM, Wu Y and Khandjian EW (1993) Tissue-specific expression of heat shock proteins of the mouse in the absence of stress. Dev. Genet. **14**, 112–118.

Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl Acad. Sci. USA **76**, 4350–4354.